FEBS Letters 586 (2012) 1306–1311

FEBS Letters

journal homepage: www.FEBSLetters.org

Loss of *mPer2* increases plasma insulin levels by enhanced glucose-stimulated insulin secretion and impaired insulin clearance in mice

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ARTICLE INFO

Article history:
Received 2 December 2011
Revised 12 March 2012
Accepted 15 March 2012
Available online 5 April 2012

Edited by Laszlo Nagy

Keywords:
mPer2
Insulin secretion
Insulin clearance
Glucose homeostasis

ABSTRACT

The existence of peripheral oscillators has been shown, and they are critically important for organizing the metabolism of the whole body. Here we show that mice deficient in *mPer2* markedly increase circulatory levels of insulin compared with wild type mice. Insulin secretion was more effectively stimulated by glucose, and alloxan, a glucose analogue, induced more severe hyperglycemia in *mPer2*-deficient mice. Hepatic insulin degrading enzyme (*Ide*) displayed an obvious day and night rhythm, which was impaired in mPer2-deficient mice, leading to a decrease in insulin clearance. Deficiency in *mPer2* caused increased *Clock* expression and decreased expression of *Mkp1* and *Ide1*, possibly underlying the observed phenotypes and suggesting that *mPer2* plays a role in regulation of circulating insulin levels.

1. Introduction

Insulin is a polypeptide hormone which is essential for the regulation of glucose metabolism, and is synthesized exclusively in β cells of the islets and degraded mainly in liver and kidney [1–2]. Circulating insulin affects many areas of metabolism, including carbohydrate, lipid, protein and mineral metabolism [3]. Insulin facilitates entry of glucose into muscle, adipose and several other tissues and stimulates liver to store glucose in the form of glycogen. Insulin decreases production of glucose from non-sugar substrates, promoted synthesis of fatty acids in the liver and inhibits breakdown of fat in adipose tissue. Gene-mutation and knockout models have identified that plasma insulin levels are regulated by insulin secretion and clearance [4–6]. It is well known that glucose stimulates insulin secretion, which is modulated by pancreatic glucose transporter 2 (*Glut2*) level. Mice lacking a functioning *Glut2* gene manifest hypoinsulinemia [4]. Insulin-degrading enzyme (*Ide*) is widely recognized as the principal protease responsible for the clearance of insulin. Mice deficient in *Ide* markedly increase levels of serum insulin [5].

Both the processes of insulin secretion and degradation are circadian controlled [7–13]. Several studies demonstrate that insulin-producing beta cells of islets among the pancreatic cell-types possess an intrinsic clock and a rhythmic pattern of the insulin secretion rates have been identified [7–12]. Human insulin secretion rates change in a circadian rhythm during 24 h [10,11]. Moreover, a circadian regulation of feeding-induced insulin responses is observed in rat, which is independent of the temporal distribution of feeding activity [12]. The diurnal variation in insulin clearance rate is also described in previous work. Human insulin clearance rate is significantly higher in the evening than in the morning [13].

The clock gene *mPer2* is an important component of the circadian system, both in the brain and in peripheral tissues [14,15]. Its functions are involved in a number of regulations of different biological process and pathways [16–19]. Mice deficient in *mPer2* reduced endurance of muscles accompanied by increased levels of glycolytic enzymes in the anterior tibialis muscle, indicating a greater dependence on anaerobic metabolism under stress conditions [16]. Also, *mPer2* mutant mice display altered lipid metabolism with a remarkable reduction in body weight and epididymal fat pad mass by direct regulation of PPARγ [17].

Whether *mPer2* influences insulin secretion and its action remains unclear. Here, we showed that plasma insulin levels were elevated *in mPer2*-deficient mice, which was associated with enhanced glucose-stimulated insulin secretion and impaired insulin clearance. Our findings suggested *mPer2* played a role in regulation of circulatory insulin levels.

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.03.034

2. Materials and methods

2.1. Animal

The *mPer2*-deficient mice used in this study were kindly provided by Dr. Lee CC [18], and were bred onto the C57BL/6J (Jackson Laboratory, Bar Harbor, ME, USA) background for eight to ten generations (N8 to N10), according to standard genetic protocols. Age and gender matched C57BL/6J animals were used as controls in all experiments. All the mice were housed in a standard animal maintenance facility under a 12-h/12-h light/dark cycle, and with free access to regular chow food and given access to standard mouse chow and water ad libitum, except when overnight fasting was required. The experiments were performed under protocols approved by the institutional animal care committee of NJUST.

2.2. Plasmid, primers and regents

All the primers were synthesized by Invitrogen (USA). D-glucose, insulin, glycogen, alloxan, L-alanine, sodium pyruvate and all inorganic salts were purchased from Sigma Inc (USA). *mPer2* cDNA Plasmids were kindly provided by Dr. Lee CC. Glycogen assay kit was purchased from Nanjing Jiancheng Bioengineering Institute. (China). Cell culture reagents were purchased form Thermo Fisher Scientific Inc (USA). MMLV reagent was purchased from Takara Bio Inc (Japan). SYBR Green Realtime PCR Master Mix was purchased from Toyobo Inc (Japan). Iodine [^{125}I] Insulin Radioimmunioassay Kit was purchased from Beijing Chemclin Biotech Co., Ltd. (China).

2.3. Glucose tolerance, insulin sensitivity and insulin secretion tests

For glucose tolerance, mice were fasted 14 h and then injected intraperitoneally (i.p) glucose 2 g/kg body weight at Zeitgeber time (ZT)2 and ZT14. Total blood glucose was determined before and after injection by One Touch Blood Glucose Meter (AW063-436-01A), (LifeScan InC) using 3 μl of whole blood obtained by tail bleed at 0, 30, 60, and 120 min. For glucose stimulated insulin secretion, glucose (2 g/kg of body weight) was injected i.p and venous blood was collected at 0, 15, 30 and 120 min. Plasma was separated and frozen in aliquots and stored at −20 °C. Plasma insulin was measured using radioimmunoassay by commercial Iodine [^{125}I] Insulin Radioimmunioassay Kit. For insulin sensitivity test, mice were fasted for 14 h and injected i.p with regular insulin (100 U/ml) resulting in a final concentration of 0.125 U/kg body weight. Blood was collected at 0, 30, 60 and 180 min after the injection and measured by One Touch Blood Glucose Meter.

2.4. Histologic analyses

Pancreas from abdominal region were fixed in 10% neutral buffered formalin for 24 h and then processed in 70% alcohol for histological analysis. Tissues were embedded in paraffin and 8 μm sections were cut and stained with hematoxylin and eosin stain (H&E) procedures. Estimation of islet number was performed by counting the number of islets per 10 μm section of four to seven sections per mouse, using three mice per genotype. The circling islet area and the area of each section were determined with the BioQuant image analyzer software package.

2.5. Determination of hepatic glycogen content

Mice were fasted 14 h and then injected intraperitoneally glucose 2 g/kg body weight at ZT2. Mice were killed at the indicted time by cervical dislocation or decapitation. Liver were rapidly excised, quick-frozen in liquid nitrogen, and stored at −80 °C. Glucogen contents were determined using glucogen assay kit.

Table 1
Primer sequences for relative genes.

Gene	Forward primer	Reverse primer
Glut2	CTGGGTCTGCAATTTTGTCA	TGTAAACAGGGTGAAGACCA
Ide	GGGAAATCATCTCGCAGCAGT	TGGCGCTTCGGACAA GTTTAT
Mkp1	TGCCTGACAGTGCAGAATCC	TCCTTCCG AGAAGCGTGATAG
Clock	CGGCGAGAACTTGGCATT	AGGAGTTGGGCTGTGATCA
β-Actin	GATCATTGCTCCTCCTGAGC	ACTCCTGCTTGCTGATCCAC

2.6. Gluconeogenesis analysis

Gluconeogensis analysis was performed as previously reported [20]. WT and *mPer2*-deficient mice were fasted 14 h and then injected 200 mg/kg L-alanine and 500 mg/kg sodium pyruvate at ZT2. Tail-vein blood samples were assessed for glucose concentration at the indicated time points (0, 15, 30, 45 and 60 min).

2.7. Alloxan treatment

WT and *mPer2*-deficient mice were fasted overnight (14 h) and then inject intraperitoneally at low dose (140 mg/kg) of alloxan. To assess the onset and severity of the diabetes, blood glucose levels from tail blood were measured 3th days after the initial injection. Total blood glucose was determined after fasting overnight.

2.8. Cell culture and plasmid transfections

HepG2 cell was maintained in DMEM supplemented with antibiotics. and 15% fetal calf serum in an atmosphere of humidified 95% air and 5% CO_2 at 37 °C. The cells were transfected with the *mPer2* cDNA plasmids indicated using transfection reagent (Lipofectamine 2000 Reagent, Invitrogen). Cells were prepared 24 h later for extracting total RNA and examination of *Ide*, *Mkp1* and *Clock* expression by real time RT-PCR.

2.9. Quantitative real time RT-PCR

Total RNA was extracted from the fresh liver and cell samples with Trizol Reagent. Islets were isolated by collagenase digestion using the protocol [21] and total mouse islet RNA was extracted as described [22]. RNA was reverse-transcribed with poly (dT)12–18 as a first-strand primer according to the manufacturer's instructions. Real-time PCR was performed and analyzed using an ABI 7300 Detection System in combination with SYBRgreen dye. The primers details and reaction conditions can be seen in Table 1. Relative expression in comparison with *β-Actin* was calculated by the comparative CT method.

3. Results

3.1. Elevation of plasma insulin level in mPer2-null mice

We measured insulin level in plasma samples collected from wild type (WT) and *mPer2*-null mice. As shown in Fig. 1A, The serum insulin level of *mPer2*-null mice was about 1.5-fold higher than WT mice while fasted for 14 h (0 point in Fig. 1A). Next we examined the glucose stimulated insulin secretion. At 15 min and 30 min after i.p. glucose injection, plasma insulin levels increased significantly more in *mPer2*-null mice compared with WT mice. Plasma insulin level returned to pre-injection levels at 120 min in both genotypes. GTT tests were then carried out in both WT

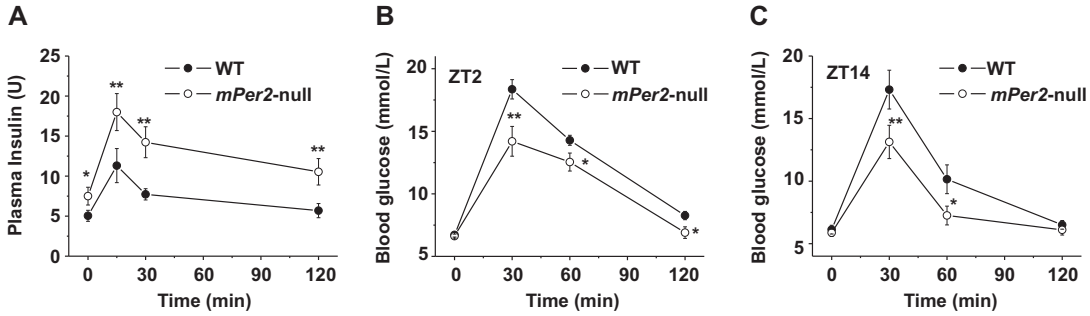

Fig. 1. Elevated plasma insulin levels and increased glucose-stimulated insulin secretion in mPer2-null mice. (A) Plasma insulin levels at the indicated times before and after glucose administration. (B) Glucose tolerance tests GTT after i.p loading with 2 g D-glucose/kg were performed on 8-week-old male animals of the indicated genotypes following a 14-h fast at ZT2. (C) Glucose tolerance tests at ZT14. Data were all shown as the mean ± S.E.M. ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$.

and *mPer2*-null mice at ZT2 (Fig. 1B) and ZT14 (Fig. 1C), and the *mPer2*-null mice displayed lower blood glucose levels compared with WT mice, which was correlated with the plasma insulin levels at the same time points after glucose stimulation, indicating an increase in plasma insulin level and glucose utilization in *mPer2*-null mice.

3.2. Increased Glucose transporter 2 (Glut2) gene expression in mPer2-null mice

To further analyze the factors contributing to enhance glucose-stimulated insulin secretion in *mPer2*-null mice, we analyzed the islet histology. Pancreatic islets of *mPer2*-null mice were indistinguishable in appearance from those of WT mice (Fig. 2A) and no significant difference was found between two genotypes in the numbers of islets (Fig. 2B) and mean size of islets (Fig. 2C). Thus it was unlikely that islet development reflected increased glucose-stimulated insulin secretion in *mPer2*-null mice. Then we analyzed the mRNA levels of *Glut2* in the isolated pancreatic islets. The quantitative RT-PCR showed that *Glut-2* mRNAs of pancreatic islets were markedly increased in the *mPer2*-null mice compared with WT mice (Fig. 2D). Alloxan is a toxic glucose analogue that preferentially accumulates in pancreatic beta cells via *Glut2*-dependent transport and is used to induce hyperglycemia. We exposed alloxan (140 mg/kg body weight) to WT and *mPer2*-null mice, respectively. As shown in Fig. 2E, the *mPer2*-null mice responded with more severe hyperglycemia compared to the wild type controls. These results reveal that enhanced glucose-stimulated insulin secretion is associated with increased *Glut2* expression in pancreatic islets of *mPer2*-null mice.

3.3. Impaired hepatic insulin degrading enzyme (Ide) gene expression and decreased insulin clearance in Per2-null mice

Reduced insulin clearance also led to elevated levels of circulating insulin, so we next examine insulin clearance in WT and *mPer2*-null mice. Insulin tolerance tests were carried out in *mPer2*-null and compared with WT mice. As shown in Fig. 3A, after an i.p injection of insulin solution at a dose of 0.125U /kg of body weight, the systemic glucose levels in WT mice and *mPer2*-null mice were both significantly reduced. The *mPer2*-null mice had similarly reduced levels of blood glucose levels 15 min post-injection, suggesting that *mPer2*-null mice had normal insulin sensitivity. However, delayed glucose recovery after insulin-induced hypoglycemia was observed in *mPer2*-null mice at subsequent time points. These results suggest that excessive insulin is still not degraded in *mPer2*-null mice and *mPer2*-null mice decrease the rate of insulin clearance (Fig. 3A). Then, we extracted liver total RNA from both genotype mice at ZT4 and ZT16 for checking hepatic *Ide* expression, which can contribute to insulin clearance. Interestingly, *Ide* mRNA expression had an obvious day and night rhythm (Fig. 3B), which was similar pattern to *mPer2* mRNA expression. In *mPer2*-null mice *Ide* levels were significantly lower compared to WT mice at both ZT4 and ZT16 (Fig. 3B); the mRNA of *Clock* and clock-controlled

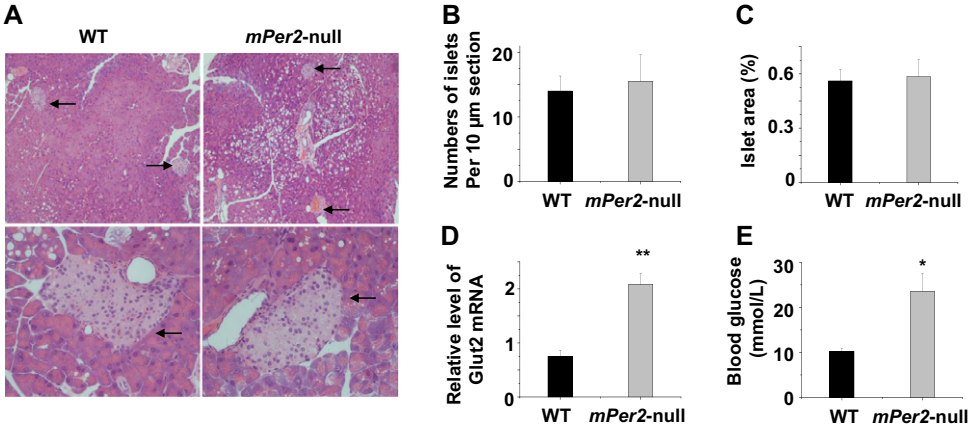

Fig. 2. Increased Glut2 expression in pancreatic islets of mPer2-null mice. (A) Pancreatic tissue was embedded in paraffin and 10 μm sections were cut and stained with hematoxylin and eosin stain (H&E) procedures, and sections were analyzed using light microscopy. Islets were indicated by arrow (original magnification, ×100 and ×400). (B) The number of Islets enumerated from microscopic examination of histological sections of pancreas from wild-type and *mPer2*-null mice expressed as the number of 10 μm of four to seven sections per mouse. (C) The islet area was measured by circling islets. (D) Quantification of *Glut2* expression in pancreatic islets from WT and *mPer2*-null mice. (E) Plasma glucose level after injection of alloxan. Data were all shown as the mean ± S.E.M. ($n = 6–8$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared to wild type mice.

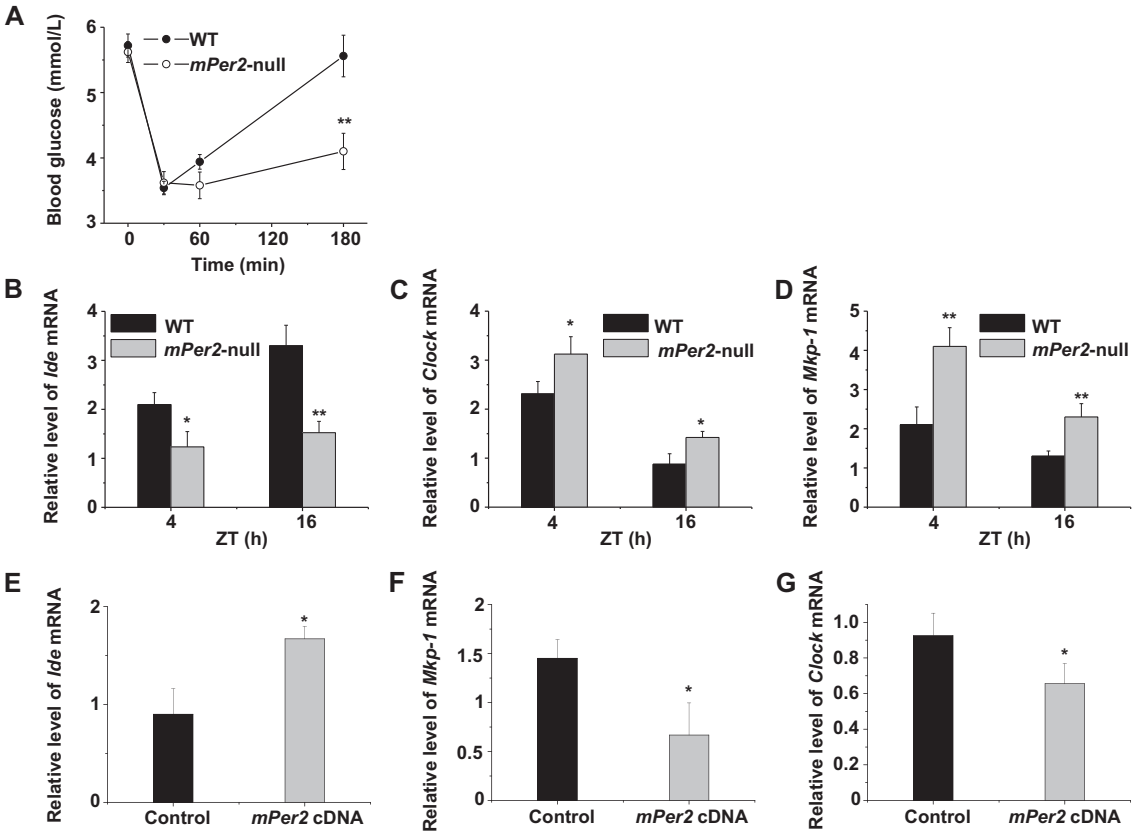

Fig. 3. Loss of mPer2 resulted in decreased insulin clearance. (A) The decline in blood glucose from basal levels of mice was monitored following injections with 0.125 units/kg insulin. *(B)*Comparison of relative level of *Ide*, (C) *Clock* and (D) *Mkp1* mRNA assessed by real-time RT-PCR in liver of *mPer2*$^{-}$null and WT mice at ZT4 and ZT16. (E) Quantitative real time RT-PCR was performed to assay *Ide*, (F) *Mkp1 and* (G) *Clock* mRNA levels. HepG2 cell lines were transfected with *mPer2* cDNA plasmid for 24 h. Relative expression in comparison with *β-Actin* was calculated by the comparative CT method. Data were all shown as the mean ± S.E.M. (n = 4–8) $^{*}P < 0.05$, $^{**}P < 0.01$.

gene *Mkp1* displayed day and night variation but reached 1.5-fold higher levels in *mPer2*-null livers than WT livers (Fig. 3C and D). Transfecting *mPer2* cDNA into culture cell lines resulted in increase in *Ide* expression (Fig. 3E), and a decrease in expression of *Mkp1 and Clock* mRNA (Fig. 3F and G).

3.4. Increased liver glycogen levels and impaired gluconeogenesis in mPer2-null mice

Elevated circulatory insulin levels results in increased liver glycogen synthesis. Therefore we examined liver glycogen in *mPer2*-null and wild type mice, respectively. As shown in Fig. 4A, *mPer2*-null mice have increased liver glycogen both under fasting conditions and in response to a bolus of injected glucose. Insulin was also the most important hormone that inhibits gluconeogenesis. Injection of L-alanine and sodium pyruvate was performed to examine gluconeogenic capacity in WT and *mPer2*-null mice. As shown in Fig. 4B and C, *mPer2*-null mice displayed a lesser increase in blood glucose in response to pyruvate or alanine injection compared to WT mice, implying that hepatic gluconeogenesis was impaired in *mPer2*-null mice.

4. Discussion

Several investigations have demonstrated that circadian system plays an important role in glucose metabolism [7,8,23,24].

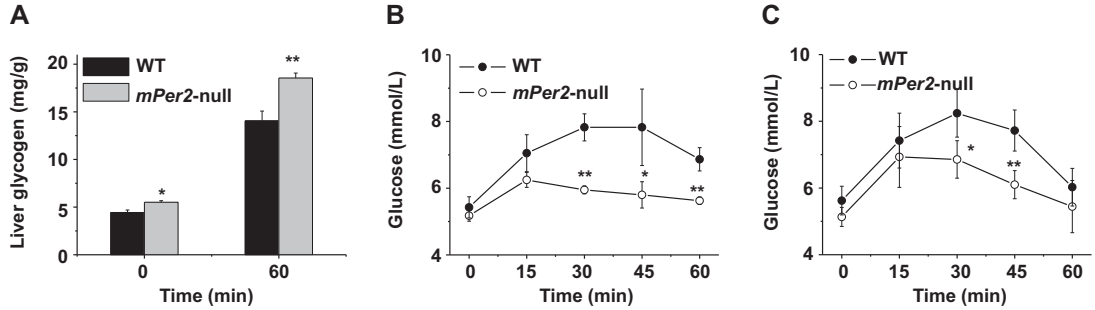

Fig. 4. Increased liver glycogen content and impaired hepatic gluconeogenesis in mPer2-null mice. (A) Liver glycogen content was measured in the fasted state and 1 h after glucose injection (60 min). (B) 14 h fasted WT and *mPer2*-null mice each received an i.p. injection 200 mg/kg L-alanine or (C) 500 mg/ kg sodium pyruvate, and tail-vein blood samples were assessed for glucose concentration immediately before injection (time 0) and at the indicated time points post injection. Data were all shown as the mean ± S.E.M. (n = 4–6). $^{*}P < 0.05$, $^{**}P < 0.01$.

Clock mutant mice display significantly elevated fasting glucose levels and impaired glucose tolerance [8]. Mice deficient in *Bmal1* also exhibit impaired glucose tolerance and abolished hepatic gluconeogenesis [8,23,24]. Both *Clock* and *Bmal1* mutant mice exhibit impaired glucose-stimulated insulin secretion [7,8]. In our present study, mice lacking in *mPer2* resulted in elevation of plasma insulin level, which increased glucose utilization and impaired gluconeogenesis. On the contrary to *Clock* and *Bmal1* knock out mice, loss of *mPer2* function in the mice enhanced glucose-stimulated insulin secretion. It is well known *mPer2* is driven by the basic helix–loop–helix–PAS protein (Clock-Bmal1) complex, which binds the E-box on the genes, and this Clock-Bmal1-mediated transcription is in turn repressed by PER2 etc. complexes that translocate to nuleus [25,26]. Thus, loss of *mPer2* function could result in enhanced *Clock* and *Bmal1* expression and increased the activation of Clock-Bmal1-mediated transcription, displaying a reverse effect on glucose-stimulated insulin secretion compared to *Clock* and *Bmal1* deficient mice. Strangely, double knockout of $mPer1^{-/-}$, $mPer2^{-/-}$ mice in a pure 129Sv/Ev genetic background were observed to impair glucose tolerance [23]. This difference could be due to the different genetic backgrounds or to the additional loss of *mPer1*.

Expression of *mPer2* is dampened in livers of insulin deficient mice (STZ-treated), which is normalized by injection of insulin [27]. In WT mice, insulin injection during the daytime causes a phase advance of hepatic *mPer2* expression rhythm, and insulin infusion induced an acute increase of *mPer2* gene expression and a similar phase advance of *mPer2* expression rhythm in vitro experiment [28]. Data presented here reveal that loss of *mPer2* increased plasma insulin level by increased pancreatic *Glut2* expression and enhanced glucose-stimulated insulin secretion. These findings suggest the existence of a crosstalk between insulin secretion and *mPer2* expression.

Plasma insulin level is also regulated by hepatic *Ide* expression [2]. *Ide*- deficient mice display hyperinsulinemia [5]. In vitro, *Ide* gene expression is decreased by inhibiting phosphor-mitogen-activated protein kinase (phospho-MAPKs) signal transduction pathways [29]. MAP kinase phosphatase 1 (MKP1), a negative regulator for MAPK-mediated signal transduction, is a Clock-controlled gene expressed in the central pacemaker neurons of the hypothalamic SCN [30]. Our results demonstrated loss of *mPer2* decreased hepatic *Ide* expression, which was related to increase *Clock* and its controlled gene *Mkp1* expression. High plasma insulin level is a risk factor for several diseases [31–33]. Elevated insulin levels are associated with hypoglycemia, cancer and vessel disease. *mPer2*-null and $Per2^{m/m}$ mice suffer multiple abnormalities, including increased carcinogenesis and impaired vascular endothelial function [34–36]. Elevated plasma insulin could play a partial role in multiple abnormalities in *mPer2*-deficient mice.

In summary, lack of *mPer2* elevated plasma insulin levels, which was associated with enhanced glucose-stimulated insulin secretion and impaired insulin clearance.

Acknowledgements

We are indebted to Dr. Lee CC for kindly providing mPer2-null mice and mPer2 cDNA plasmid. This work was supported by the 973 Program of China (2012CB517505) and the National Science Foundation of China (31071130).

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